Cell Stem Cell
Short Article

The Nuclear Receptor Nr5a2 Can Replace Oct4 in the Reprogramming of Murine Somatic Cells to Pluripotent Cells

Jian-Chien Dominic Heng,[1,4,7] Bo Feng,[1,7] Jianyong Han,[2,7] Jianming Jiang,[1] Petra Kraus,[2] Jia-Hui Ng,[1,4] Yuriy L. Orlov,[3] Mikael Huss,[3] Lin Yang,[1] Thomas Lufkin,[2,5] Bing Lim,[2,6] and Huck-Hui Ng[1,4,5,*]
[1]Gene Regulation Laboratory
[2]Stem Cell and Developmental Biology
[3]Computational and Systems Biology Group
Genome Institute of Singapore, Singapore 138672, Singapore
[4]NUS Graduate School for Integrative Sciences and Engineering, Singapore 117597, Singapore
[5]Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore
[6]Center for Life Sciences, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02115, USA
[7]These authors contributed equally to this work
*Correspondence: nghh@gis.a-star.edu.sg
DOI 10.1016/j.stem.2009.12.009

SUMMARY

Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) with the introduction of Oct4, Sox2, Klf4, and c-Myc. Among these four factors, Oct4 is critical in inducing pluripotency because no transcription factor can substitute for Oct4, whereas Sox2, Klf4, and c-Myc can be replaced by other factors. Here we show that the orphan nuclear receptor Nr5a2 (also known as Lrh-1) can replace Oct4 in the derivation of iPSCs from mouse somatic cells, and it can also enhance reprogramming efficiency. Sumoylation mutants of Nr5a2 with enhanced transcriptional activity can further increase reprogramming efficiency. Genome-wide location analysis reveals that Nr5a2 shares many common gene targets with Sox2 and Klf4, which suggests that the transcription factor trio works in concert to mediate reprogramming. We also show that Nr5a2 works in part through activating Nanog. Together, we show that unrelated transcription factors can replace Oct4 and uncovers an exogenous Oct4-free reprogramming code.

INTRODUCTION

The reversion of somatic cells to pluripotent cells is commonly referred to as reprogramming. Somatic cell nuclear transfer and cell fusion are well-known examples of techniques employed in the reprogramming of differentiated cells (Lewitzky and Yamanaka, 2007). Another method of reprogramming was discovered when Takahashi and Yamanaka reverted mouse embryonic fibroblasts (MEFs) into pluripotent cells known as iPSCs with the retroviral introduction of four transcription factors Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Since the discovery of iPSCs, cells from different somatic lineages and a diverse range of species have been successfully reprogrammed (Feng et al., 2009b). Besides the four reprogramming factors discovered in the groundbreaking study of Yamanaka, other factors such as NANOG and LIN28 were also found to participate in reprogramming (Yu et al., 2007). Recently, Esrrb, an orphan nuclear receptor, was shown to be a reprogramming factor capable of replacing Klf4 in the reprogramming of MEFs (Feng et al., 2009a). Close family members of reprogramming factors are also capable of replacing their counterparts. For example, Klf2 and Klf5 can replace Klf4; Sox1 and Sox3 can substitute for Sox2; and c-Myc can be replaced by N-Myc and L-Myc (Nakagawa et al., 2008). However, to date, Oct4 remains irreplaceable by other transcription factors including its close family members Oct1 and Oct6 (Nakagawa et al., 2008). Oct4 is expressed in the inner cell mass (ICM) of developing blastocysts and is critical for maintaining pluripotency in cells of the ICM and embryonic stem cells (ESCs) (Nichols et al., 1998; Schöler et al., 1990). Although neural progenitor cells (NPCs) express high levels of endogenous Sox2, ectopic expression of Oct4 was still required for their reprogramming (Kim et al., 2009). This observation suggests that Oct4 is pivotal in imparting pluripotency to somatic cells. Hence, it will be interesting to uncover other transcription factors that could replace Oct4 in the reprogramming of somatic cells. In addition, only a few transcription factors such as Oct4 and the aforementioned transcription factors have been reported to contribute to iPSC generation. Therefore, to expand the regulatory code for reprogramming, we embarked on a screen to identify new reprogramming factors. We identified two nuclear receptors that can enhance reprogramming. Interestingly, one of these factors, Nr5a2, can replace exogenous Oct4 and works in conjunction with Sox2 and Klf4.

RESULTS AND DISCUSSION

Screen of Nuclear Receptors Reveals that Nr1i2 and Nr5a2 Can Enhance Reprogramming Efficiency

We carried out a screen of 19 nuclear receptors (Table S1 available online) for their ability to enhance reprogramming efficiency.

MEFs containing a *Pou5f1-GFP* reporter (Figure S1A; Feng et al., 2009a) were used to identify putative iPSC colonies, based on the reactivation of the *Pou5f1* promoter. We transfected each nuclear receptor retrovirally along with Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M) retroviruses. The frequency of GFP-positive colonies was determined at 14 days postinfection (dpi). Transcript expression of all the nuclear receptor constructs was verified (data not shown). From this screen, we found that both orphan nuclear receptors, Nr1i2 (also known as pregnane X receptor, Pxr) and Nr5a2 (also known as liver receptor homolog-1, Lrh-1), can enhance the efficiency of reprogramming (as compared to OSKM control) by 2.7-fold and 4.0-fold, respectively (Figure 1A). Addition of Nr5a2 also enhanced the kinetics of OSKM reprogramming with GFP expression detectable 3 days earlier than in the case of conventional four factor reprogramming (Figure 1B). Cell viability assays confirmed that both Nr1i2 and Nr5a2 do not induce cell death (Figure S1B).

Nr5a2 Can Replace Oct4 in the Reprogramming of MEFs to iPSCs

We next investigated whether Nr1i2 and Nr5a2 could replace the core reprogramming factors in addition to enhancing reprogramming efficiencies. c-Myc has already been demonstrated to be dispensable for reprogramming (Nakagawa et al., 2008; Wernig et al., 2008), so we did not investigate the replaceability of c-Myc but instead tested the ability of these two nuclear receptors in replacing any of the OSK trio. Nr1i2 was unable to replace O, S, or K, and Nr5a2 was unable to replace S or K (Figure 1C). Strikingly, when *Pou5f1*-GFP MEFs were transduced with Nr5a2 and SKM viruses, GFP-positive colonies (23.7 ± 3.5 per 100,000 MEFs plated) were observed by 14 dpi (Figure 1C; Figures S1C and S1D). This demonstrates that besides augmenting reprogramming efficiency, exogenous Nr5a2 could also replace exogenous Oct4. We refer to these cells that have been reprogrammed with Nr5a2, Sox2, Klf4, and c-Myc as N_2SKM iPSCs. These colonies could be stably passaged long-term and stained positive for alkaline phosphatase (Figure S1E), Nanog (Figures S1F and S1G), and SSEA-1 (Figures S1H and S1I). The other 18 nuclear receptors were also tested for their ability to replace Oct4. However, unlike Nr5a2, none were able to replace Oct4 (Figure S1J).

Given that c-Myc is dispensable for reprogramming, we were also able to generate iPSCs from *Pou5f1-GFP* MEFs that were transduced with Nr5a2 and SK viruses, albeit at a lower efficiency (2.3 ± 0.6 per 100,000 MEFs plated) than that of N_2SKM combination (Figures 1D–1F). These three-factor Nr5a2-reprogrammed cells are referred to as N_2SK iPSCs. Similar to N_2SKM iPSCs, N_2SK iPSCs stained positive for alkaline phosphatase (Figure 1G), Nanog (Figures 1H and 1I), and SSEA-1 (Figures 1J and 1K).

Nr5a2-reprogrammed cells were karyotypically normal (Figure S1K) and genomic integrations of the respective viruses into the genomic DNA were verified and showed no evidence of *Pou5f1* transgene integration (Figure S1L). Both embryoid body-mediated differentiation and teratoma formation assays were carried out to test the pluripotency of the Nr5a2-reprogrammed cells. Nr5a2-reprogrammed cells were indeed pluripotent as shown by the fact that they could be in vitro differentiated into cells of the three major germ layers (Figure S2A) and form teratomas that consisted of differentiated tissue originating from the three major germ layers (Figure S2B).

A more stringent assay for pluripotency was performed whereby Nr5a2-reprogrammed cells were microinjected into 8-cell stage wild-type C57BL/6J or B6(Cg)-Tyr^{c-2J}/J (B6-albino) embryos. The Nr5a2-reprogrammed cells were derived from *Pou5f1-GFP* MEFs, so E13.5 embryos displayed GFP expression in the gonads because of high levels of endogenous Oct4 expression (Figures 1L and 1M). In addition, live-born chimeras were generated from both N_2SKM (Figure S1M) and N_2SK (Figure 1N) lines. More importantly, the N_2SK line is germline competent (Figure 1O; Table S2).

Expression and Epigenetic Profiling of Nr5a2-Reprogrammed Cells Closely Resemble ESCs

Global gene expression profiling of Nr5a2-reprogrammed cells was performed and hierarchical clustering of the microarray data revealed that Nr5a2-reprogrammed cells were more similar to ESCs and OSKM iPSCs than MEFs (Figure S2C). In addition, expression profiling showed a concomitant upregulation of ESC-associated genes and a downregulation of MEF-associated genes in Nr5a2-reprogrammed cells (Figure S2D).

Next, promoter methylation analysis revealed that the *Pou5f1* and *Nanog* promoters of Nr5a2-reprogrammed cells were largely unmethylated (Figure S2E) and were similar to that of ESCs. We also explored the bivalent domain patterns of Nr5a2-reprogrammed cells. Our results indicated that Nr5a2-reprogrammed cells possessed both active H3K4me3 and repressive H3K27me3 chromatin modifications (Figure S2F) that were similar to that of ESCs.

The Close Family Member Nr5a1 Can Also Enhance Reprogramming Efficiency and Replace Oct4

Closely related members of the same family of transcription factors can replace each other in the context of reprogramming (Feng et al., 2009a; Nakagawa et al., 2008). Because both Nr5a1 (also known as steroidogenic factor 1, Sf1) and Nr5a2 belong to the same nuclear receptor subfamily V, we were interested to examine whether Nr5a1 was able to both enhance the efficiency of reprogramming and replace Oct4. Nr5a1 enhanced reprogramming efficiency (Figure 2A) but to a lesser extent than Nr5a2 (Figure 1A). Next, we investigated whether Nr5a1 could replace any of the core reprogramming factors (O, S, and K). Similar to Nr5a2, Nr5a1 was unable to replace S and K but it was able to replace Oct4 (Figure 2B). We refer to these GFP-positive iPSC colonies (Figures 2C and 2D) as N_1SKM iPSCs. These Nr5a1-reprogrammed cells express alkaline phosphatase (Figure 2E), Nanog (Figures 2F and 2G), and SSEA-1 (Figures 2H and 2I). We verified the genomic integration of viral *Nr5a1* in N_1SKM iPSCs and found no evidence of viral *Pou5f1* and viral *Nr5a2* genomic integrations (Figure 2J). These karyotypically normal N_1SKM iPSCs (Figure 2K) could be differentiated in vitro to lineages of the three different germ layers (Figure 2L) and form teratomas comprising tissues of the three different lineages (Figure 2M). The demonstration of reprogramming with Nr5a1 shows that both members of the Nr5a subfamily indeed possess similar reprogramming properties.

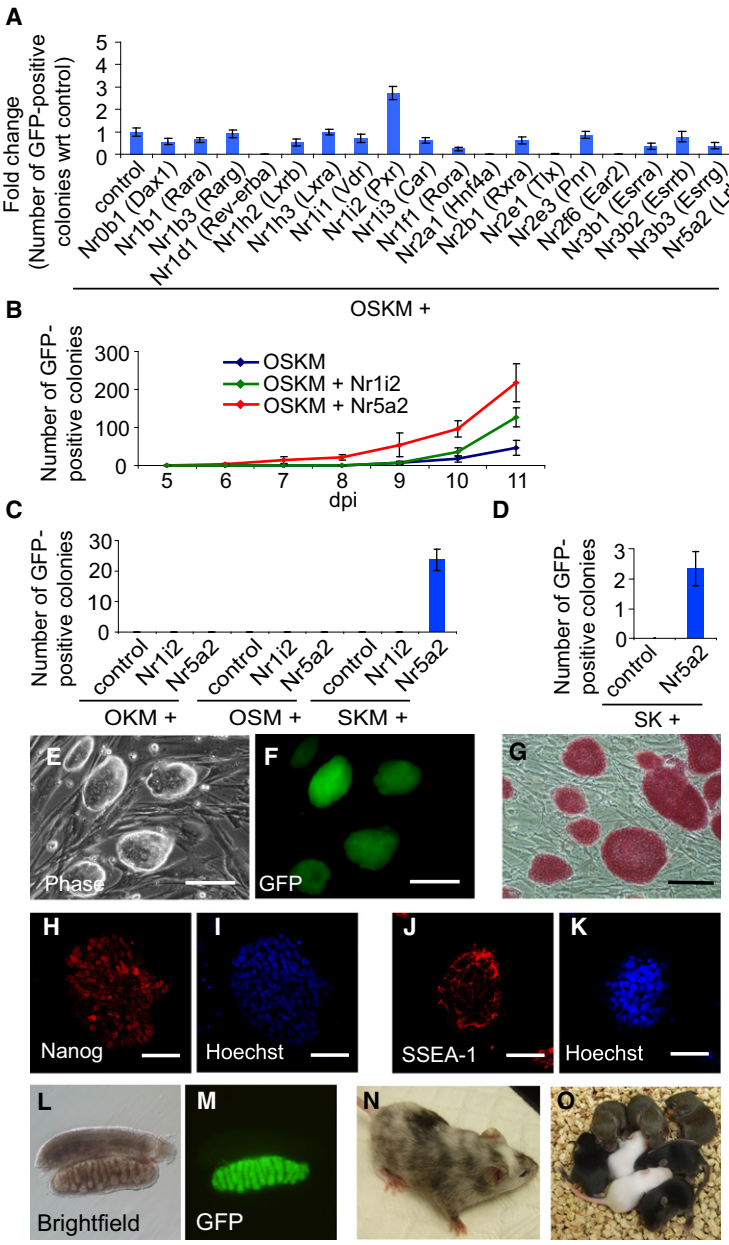

Figure 1. Nr5a2 Reprograms MEFs with Sox2, Klf4, and with or without c-Myc

(A) Screen of 19 nuclear receptors for the enhancement of OSKM reprogramming. Graph depicts fold change of number of GFP-positive colonies generated from each nuclear receptor together with OSKM with respect to OSKM (control).

(B) Kinetics of OSKM reprogramming with either Nr5a2 or Nr1i2.

(C) Reprogramming assay of reprogramming enhancers Nr1i2 and Nr5a2 for their ability to replace Sox2, Klf4, and Oct4. For control experiments, the respective combinations of retroviruses were added without Nr5a2 or Nr1i2.

(D) Number of GFP-positive colonies generated from the reprogramming of MEFs with Nr5a2, Sox2, and Klf4. For control experiment, only SK retroviruses were introduced. Data in (A)–(D) represent mean ± SEM of three retrovirus-mediated transduction experiments (n = 3).

(E) Generation of iPSC colonies after retroviral transduction of *Pou5f1-GFP* MEFs with Nr5a2, Sox2, and Klf4. Phase contrast image is shown.

(F) Colonies in (E) are GFP positive when viewed under a fluorescence microscope, indicating the reactivation of *Pou5f1* reporter.

(G) N_2SK iPSCs express alkaline phosphatase.

(H) Expression of Nanog in N_2SK iPSCs.

(I) Nuclei in (H) were counterstained with Hoechst.

(J) SSEA-1 expression in N_2SK iPSCs.

(K) Cells in (J) were stained with Hoechst to indicate nuclei. Scale bars represent 200 μm in (E)–(G) and 50 μm in (H)–(K).

(L) Brightfield image of the male gonad dissected from the E13.5 N_2SK #B3 chimeric embryo.

(M) GFP fluorescence image of (L). Positive GFP signals were observed in the gonads, indicating germline incorporation of Nr5a2-reprogrammed cells.

(N) N_2SK #B11 adult chimera generated from Nr5a2-reprogrammed cells derived from F1 (129S2/SV × *Pou5f1-GFP*) MEFs that were microinjected into B6(Cg)-Tyr^{c-2J}/J embryos.

(O) Offsprings generated from the mating of N_2SK #B11 adult chimera with an albino B6(Cg)-Tyr^{c-2J}/J mouse. Agouti and black offsprings are indicative of germline transmission of the Nr5a2-reprogrammed cells.

Other Transcription Factors that Bind *Pou5f1* Regulatory Regions Are Unable to Replace Exogenous Oct4 in Reprogramming

Nr5a2 has been shown to bind both the proximal enhancer and proximal promoter regions of *Pou5f1* and regulate *Pou5f1* in the epiblast stage of mouse embryonic development (Gu et al., 2005). *Nr5a2* null embryos display a loss of Oct4 expression in the epiblasts (Gu et al., 2005) and die between E6.5 and E7.5 (Gu et al., 2005; Paré et al., 2004). Therefore, part of the mechanism of Nr5a2 in replacing exogenous Oct4 may be explained by the findings that Nr5a2 directly regulates *Pou5f1* and acts upstream of *Pou5f1*.

We went on to investigate whether other transcription factors that bind to the *Pou5f1* promoter or enhancer region could also replace Oct4 in the reprogramming of MEFs. Therefore, we tested nine other transcription factors (Nanog, Sall4, Stat3, Zfx, Tcfcp2l1, Klf2, Klf5, N-Myc, Esrrb) that bind to the *Pou5f1* regulatory regions (Chen et al., 2008). Expression of the respective viral transcripts was verified (Figure 2N). Our results revealed that none of these transcription factors was able to replace Oct4 in the SKM combination (Figure 2O). This result shows that not all transcription factors that bind to the *Pou5f1* regulatory regions can replace Oct4 in reprogramming. Hence, Nr5a2 and its close family member, Nr5a1, are unique in their ability to replace Oct4.

DNA Binding Ability of Nr5a2 Is Important for Its Role in Reprogramming whereas Ligand Binding Is Dispensable

Similar to other nuclear receptors, Nr5a2 possesses a ligand binding domain (LBD) and a DNA binding domain (DBD). However, being an orphan nuclear receptor, the endogenous ligands of Nr5a2 remain unknown. We investigated the functional importance of ligand binding and DNA binding of Nr5a2 in reprogramming without Oct4. We mutated a specific residue to a bulkier

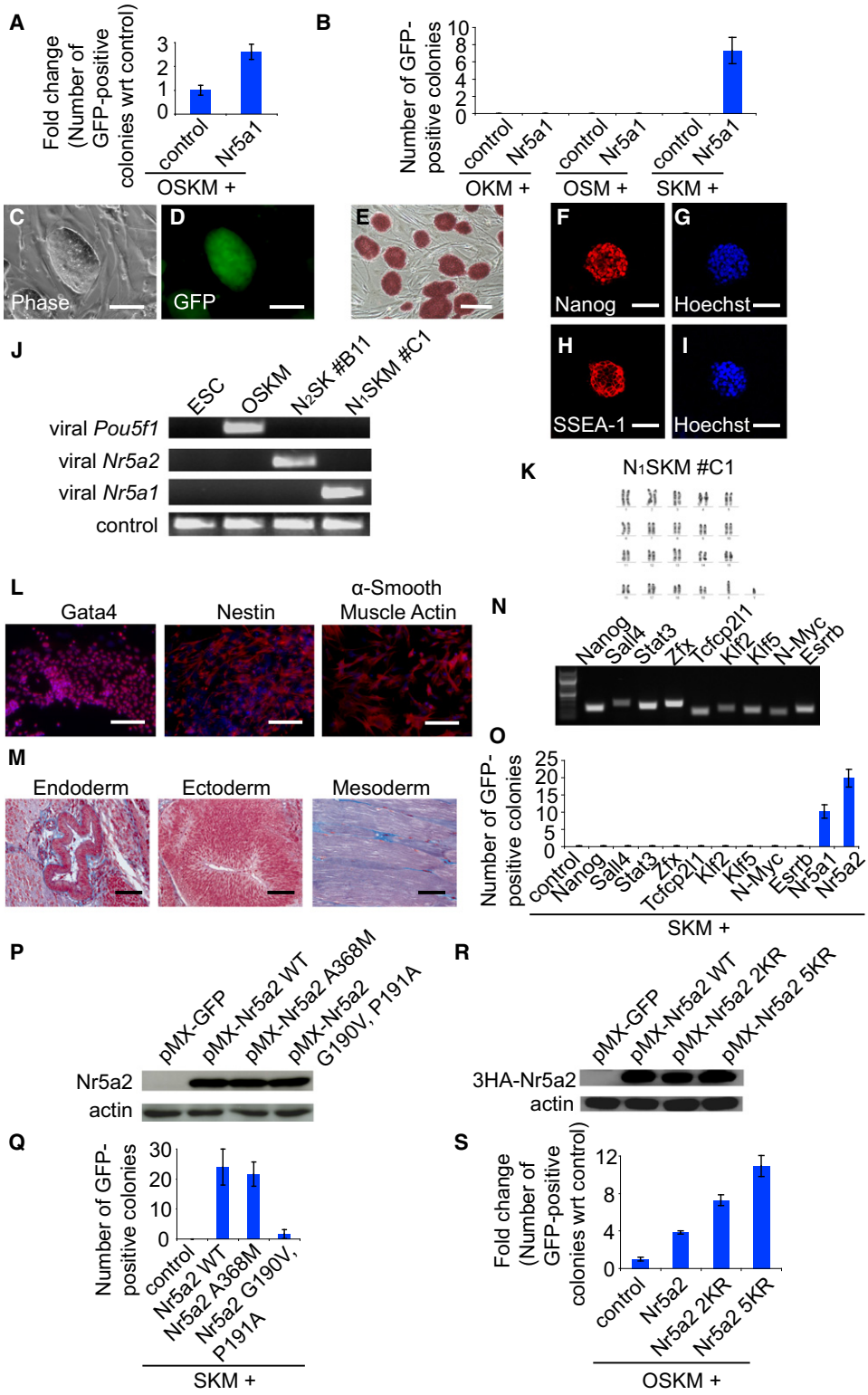

Figure 2. Nr5a1-Mediated Reprogramming and the Effect of Mutations on Reprogramming Capability of Nr5a2

(A) Nr5a1 enhances the reprogramming efficiency with OSKM. Graph depicts fold change of number of GFP-positive colonies generated from Nr5a1 in conjunction with OSKM with respect to the control (OSKM).

(B) Nr5a1 replaces Oct4 in the reprogramming of MEFs. Nr5a1 was investigated for its ability to replace Sox2, Klf4, and Oct4 by cotransducing Nr5a1 in conjunction with OKM, OSM, or SKM, respectively. Control experiments were performed with OKM, OSM, or SKM retroviruses in the absence of Nr5a1.

(C) Phase contrast image of iPSC colonies generated from the retroviral transduction of *Pou5f1-GFP* MEFs with Nr5a1 and SKM.

residue (A368M) that fills the cavity of Nr5a2 LBD so as to disrupt the binding of putative ligands (Sablin et al., 2003). Next, we created a DNA binding mutant with a double mutation (G190V, P191A) in the conserved Ftz-F1 domain that would result in a marked decrease in the DNA binding activity of Nr5a2 (Solomon et al., 2005). Western analysis was performed to ensure that these retroviral vectors expressed equivalent levels of Nr5a2 protein (Figure 2P). Our reprogramming assays show that the Nr5a2 ligand binding mutant did not decrease the number of formed GFP-positive colonies as compared to wild-type (WT) Nr5a2 (Figure 2Q). This suggests that the ability of Nr5a2 to function as a reprogramming factor is independent of ligand binding. In contrast, there was a dramatic reduction in the number of GFP-positive colonies when the Nr5a2 DNA binding mutant was introduced with SKM (Figure 2Q). Taken together, we show that the DNA binding is crucial for the reprogramming function of Nr5a2 whereas ligand binding is dispensable.

Nr5a2 Sumoylation Site Mutants Exhibit Enhanced Reprogramming Capacity

It was previously shown that when important lysine residues in Nr5a2 which could be conjugated to SUMOs are mutated, transcriptional activity increases and its localization to transcriptionally inactive nuclear bodies is impeded (Yang et al., 2009). Hence, we were interested in testing the reprogramming capacity of Nr5a2 with mutated lysine residues, by using a mutant construct with two lysine residues mutated (2KR) and another with five lysine residues mutated (5KR) (Yang et al., 2009). Western analysis showed that the WT and mutant constructs expressed similar levels of protein (Figure 2R). Strikingly, the OSKM reprogramming assay revealed that the 2KR mutant boosted reprogramming efficiency to at least 7-fold as compared to the 4-fold enhancement achieved by the WT (Figure 2S). When the 5KR mutant was introduced, reprogramming efficiency was further augmented to almost 11-fold (Figure 2S). These results suggest that the concomitant prevention of subcellular localization and the enhanced transcriptional activity brought about by the SUMO site mutations could trigger a greater induction of reprogramming by Nr5a2.

Genome-wide Binding Analysis of Nr5a2 in ESCs

Other than *Pou5f1* (Gu et al., 2005), there is no known target gene for Nr5a2 in pluripotent cells. To this end, we performed a genome-wide mapping study of Nr5a2 in ESCs by employing chromatin immunoprecipitation sequencing (ChIP-seq) technology (Table S3). We created a stable ESC line expressing HA-tagged Nr5a2, and the expression of HA-tagged Nr5a2 protein was verified by western blot with a Nr5a2-specific antibody (Figure S3A). Nr5a2-bound chromatin was enriched with an HA-tag antibody. We used the de novo motif discovery algorithm MEME and uncovered a known Nr5a2 motif enriched in our data set (Figure 3A). More importantly, our pairwise co-occurrence analyses revealed that Nr5a2 tends to colocalize with Nanog, Oct4, Sox2, Smad1, and Esrrb (Figure 3B). This result associates Nr5a2 with the previously reported Nanog-Oct4-Sox2 cluster (Chen et al., 2008). Because Nr5a2 works in concert with Sox2 and Klf4 to reprogram MEFs to iPSCs, we investigated whether these three transcription factors share similar binding targets. Interestingly, we found that all three transcription factors bind target genes that are pivotal for maintenance of ESC identity such as *Pou5f1*, *Nanog*, *Tbx3*, *Klf2*, and *Klf5* (Figure 3C; Figure S3B).

Nanog Is a Target of Nr5a2

Nanog is important in ESCs because it governs the gateway to a ground state level of pluripotency (Silva et al., 2009). To confirm that *Nanog* is a target of Nr5a2 during reprogramming, we introduced exogenous HA-tagged Nr5a2 into MEFs. ChIP experiment showed that Nr5a2 was indeed bound to the *Nanog* enhancer during reprogramming (Figure 4A). Because *Nanog* is a target of Nr5a2 in both ESCs and MEFs (Figure S3B; Figure 4A), we investigated the role of Nanog in the context of

(D) GFP-positive N_1SKM iPSC colonies in (C).
(E) Nr5a1-reprogrammed cells stained positive for alkaline phosphatase.
(F) Nanog expression in Nr5a1-reprogrammed cells.
(G) Hoechst staining of (F) indicates nuclei.
(H) Nr5a1-reprogrammed cells stained positive for SSEA-1.
(I) Hoechst staining of (H) indicates nuclei.
(J) PCR verification of genomic integration of retroviral gene *Nr5a1* in a N_1SKM line. The control panel shows PCR amplification of a region of the *p21* gene.
(K) Normal karyotype of a Nr5a1-reprogrammed line.
(L) EB-mediated in vitro differentiation assay performed on Nr5a1-reprogrammed cells. Differentiated cells stained positive for Gata4 (endoderm), Nestin (ectoderm), and α-Smooth Muscle Actin (mesoderm). Lineage markers were stained red and nuclei were stained blue with Hoechst.
(M) Teratoma assay of Nr5a1-reprogrammed cells.
Scale bars represent 200 μm in (C)–(E), 100 μm in (L), and 50 μm in (F)–(I), (M).
(N) PCR verification of viral transcript expression of *Nanog*, *Sall4*, *Stat3*, *Zfx*, *Tcfcp2l1*, *Klf2*, *Klf5*, *N-Myc*, and *Esrrb*.
(O) Screen of transcription factors that bind to *Pou5f1* regulatory regions in combination with SKM. Control represents transduction of only SKM viruses into MEFs. Nr5a1 and Nr5a2 with SKM were used as positive controls.
(P) Western analysis of cell extracts harvested from 293-T cells transfected with either retroviral vectors encoding Nr5a2 WT, Nr5a2 A368M, and Nr5a2 G190V, P191A. 293-T cells transfected with retroviral vector harboring the *GFP* gene was used as a negative control.
(Q) SKM reprogramming with Nr5a2 ligand and DNA binding mutants. *Pou5f1-GFP* MEFs were transduced with SKM viruses and viruses encoding either Nr5a2 WT, Nr5a2 A368M, or Nr5a2 G190V, P191A. Control experiment denotes infection of MEFs with only SKM viruses.
(R) Western analysis of cell extracts harvested from 293-T cells transfected with either retroviral vectors encoding Nr5a2 WT, Nr5a2 2KR, and Nr5a2 5KR. 293-T cells transfected with retroviral vector not harboring any gene was used as a negative control.
(S) OSKM reprogramming with Nr5a2 SUMO mutants. Control experiment denotes infection of MEFs with only OSKM viruses. Graph depicts fold change of number of GFP-positive colonies generated from the infection of *Pou5f1-GFP* MEFs with OSKM viruses and viruses encoding either Nr5a2 WT, Nr5a2 2KR, or Nr5a2 5KR with respect to that of the control.
Data in (A), (B), (O), (Q), and (S) represent mean ± SEM of three retrovirus-mediated transduction experiments (n = 3).

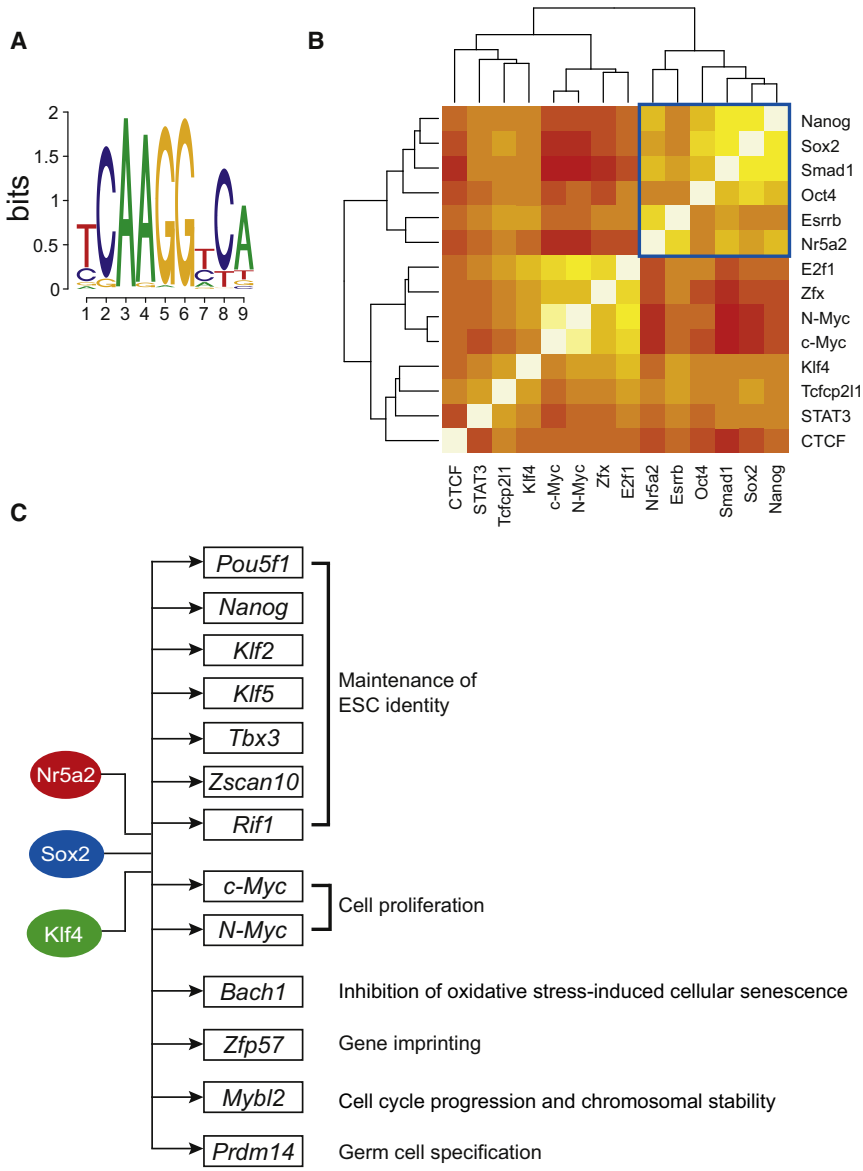

Figure 3. Genome-wide Mapping of Nr5a2 Binding Sites

(A) Motif of Nr5a2 generated by the de novo motif discovery algorithm MEME, which scans for over-represented sequences of Nr5a2-bound sites.

(B) Heat map depicting the co-occurrence of Nr5a2 and other transcription factors. Each square in the heat map denotes the frequency of colocalization between two transcription factors (red represents less frequently colocalized and yellow represents more frequently colocalized). Transcription factors have been clustered along both axes based on the similarity in their colocalization with other factors. Transcription factors demarcated by the blue box tend to colocalize with Nr5a2.

(C) Genes important in various cellular roles such as maintenance of ESC identity and cell proliferation that are bound by Nr5a2, Sox2, and Klf4.

reprogramming that involves Nr5a2. We found that *Nanog* expression increases when Nr5a2 was introduced to reprogramming MEFs (Figure 4B). As expected, endogenous *Pou5f1* increases in a similar trend as *Nanog* when Nr5a2 is introduced with OSKM during reprogramming (Figures 4C and 4D). Because Nanog is important in the transition toward the pluripotent state (Silva et al., 2009), the enhancement of reprogramming efficiency brought about by the introduction of Nr5a2 may be in part facilitated by Nanog.

Next, we wanted to know whether Nr5a2 was important in the reprogramming of MEFs. We performed a knockdown of *Nr5a2* concurrently with the introduction of OSKM to MEFs. The *Nr5a2* shRNA knockdown construct was able to reduce the *Nr5a2* mRNA and protein expression in mouse ESCs (Figures 4E and 4F). Depletion of Nr5a2 during reprogramming resulted in a reduction in the number of colonies (Figure 4G). Importantly, exogenous Nanog was able to rescue the reduction of colonies caused by the knockdown of *Nr5a2* (Figure 4G). Unlike Nanog, Mtf2, an independent factor, was not able to rescue the reduction in colonies (Figure 4G). Though Nanog was able to rescue the reduction in reprogramming efficiency brought about by Nr5a2 knockdown, Nanog was unable to rescue the effects of *Pou5f1* knockdown (Figure 4G). Interestingly, addition of both Nanog and Nr5a2 with OSKM was able to produce more GFP-positive colonies than Nr5a2 alone with OSKM (Figure 4H). Taken together, these results suggest that *Nanog* is one of the important downstream targets of Nr5a2 in the reprogramming of MEFs in which it mediates the enhancement of reprogramming efficiency.

Herein, we show that reprogramming with Nr5a2 or Nr5a1 is able to bypass the need for exogenous Oct4. Our data indicate that Nr5a2 functions synergistically with Sox2 and Klf4 to replace exogenous Oct4 to mediate the successful reprogramming of MEFs. Other than MEFs, we were also able to reprogram mouse NPCs with exogenous Nr5a2 together with Klf4 and c-Myc (data not shown). Besides being an upstream activator of *Pou5f1* (Gu et al., 2005), Nr5a2 also works in part through Nanog, an important mediator of ground state pluripotency in ESCs (Silva et al., 2009), and *Nanog* induction by Nr5a2 facilitates the acquisition of pluripotency. Recently, it was found that chemicals that inhibit Tgf-β signaling (Ichida et al., 2009; Maherali and Hochedlinger, 2009) induce Nanog to replace exogenous Sox2 in the reprogramming of MEFs (Ichida et al., 2009). Hence, Nanog is indeed an important target of reprogramming.

In summary, our study provides an example of exogenous Oct4-free code for the reprogramming of somatic cells. We also show that both Nr5a2 and Nr5a1 are able to enhance the efficiency of reprogramming with the conventional four factors.

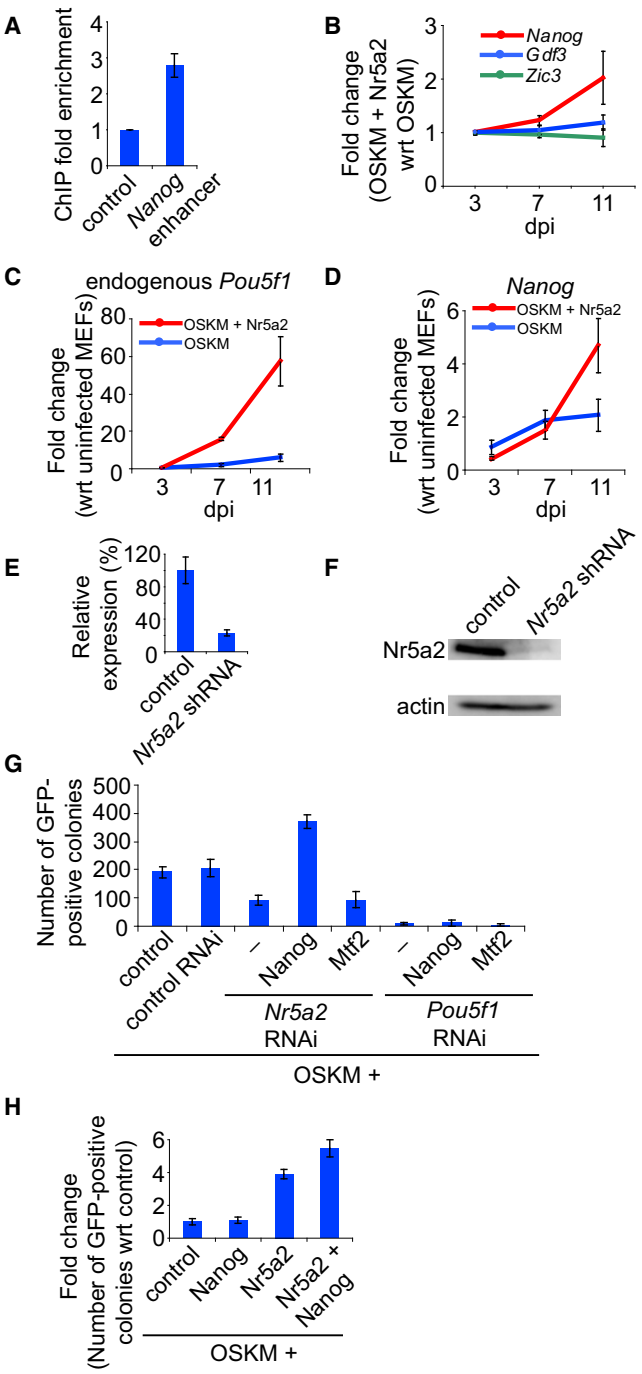

Figure 4. *Nanog* Is a Downstream Target of Nr5a2 in Reprogramming

(A) Nr5a2 binds to the *Nanog* enhancer during the reprogramming of MEFs. ChIP assay was performed on MEFs 8 days after being cotransduced with OSKM and HA-Nr5a2 viruses. Quantitative real-time PCR was performed to analyze the enrichment of HA-Nr5a2 on the *Nanog* enhancer with an HA antibody. Data shown are mean ± SEM of biological duplicates.

(B) Fold change in expression levels of *Nanog* in OSKM + Nr5a2 reprogramming cells as compared to OSKM reprogramming cells based on time-course (3, 7, and 11 dpi) biological triplicate microarray data (mean ± SEM). Fold change in expression levels of ESC-relevant genes *Gdf3* and *Zic3* were also included in the graph.

(C) Time-course fold change of endogenous *Pou5f1* mRNA levels in OSKM + Nr5a2 or OSKM-infected MEFs with respect to uninfected MEFs.

(D) Time-course fold change of *Nanog* mRNA levels in OSKM + Nr5a2 or OSKM-infected MEFs with respect to uninfected MEFs.

Real-time quantitative PCR data in (C) and (D) are mean ± SEM of biological triplicate samples.

(E) Real-time quantitative PCR verification of Nr5a2 mRNA level in ESCs after *Nr5a2* shRNA knockdown. Control ESCs were transfected with a shRNA construct targeting the *luciferase* gene.

(F) Western analyses of Nr5a2 protein expression in ESCs after introduction of knockdown construct targeting *Nr5a2*. Nr5a2 protein was targeted with an antibody specific to Nr5a2.

(G) shRNA knockdown of *Nr5a2* in OSKM reprogramming. *Pou5f1* RNAi with OSKM was used as a positive knockdown control while *luciferase* RNAi was used as a negative knockdown control. Nanog or Mtf2 was introduced to investigate their ability to rescue the knockdown effects.

(H) Reprogramming with OSKM in addition to both Nr5a2 and Nanog. Graph depicts fold change of number of GFP-positive colonies with respect to control.

Data in (E), (G), and (H) are mean ± SEM of three independent experiments (n = 3).

Altogether, we have uncovered an unexpected dual role of nuclear receptors in both enhancing and mediating reprogramming.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

iPSCs were cultured on mitomycin C-treated MEF feeders as previously described (Feng et al., 2009a). MEFs were isolated from E13.5 embryos and cultured as described previously (Feng et al., 2009a). 293-T cells were transfected with each pMX retroviral vector with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For RNAi experiments, shRNA constructs that were cloned into the pSUPER.puro vector were transfected with lipofectamine into ESCs. Cells were selected with 1 μg ml^{-1} of puromycin 16 hr posttransfection. shRNA sequences are *Pou5f1*, 5′-GAAGGATGTGGTTCGAGTA-3′; *luciferase*, 5′-GATGAAATGGGTAAGTACA-3′; and *Nr5a2*, 5′-GCAAGTGTCTCAATTTAAA-3′.

ChIP-seq Analysis

Peak calling of the Nr5a2 ChIP-seq data (8,023,427 uniquely mapped tags) was carried out with MACS with a p value cutoff of 1e-9, and 3,346 peaks were generated. The control anti-HA ChIP-seq library contained 13,001,272 uniquely mapped tags. Enriched motifs were identified by the de novo motif discovery tool MEME with 200 bp sequences centered on the ChIP-seq peaks. Co-occurrence analysis to study overlap of Nr5a2 binding sites with binding sites of other important transcription factors was performed with Nr5a2 ChIP-seq data and data set generated from our previous study (Chen et al., 2008).

Microarray Analysis

Reverse transcription of mRNA harvested from mouse ESCs, iPSCs (OSKM, N_2SKM #A5, N_2SK #B3, and #B11), and MEFs (*actin-GFP* and *Pou5f1-GFP*) was performed. Two biological replicate microarray data was generated for each cell line. For microarray of OSKM + Nr5a2 and OSKM samples, biological triplicates were used. Arrays (Sentrix Mouse-6 Expression BeadChip version 1.1) processed according to the manufacturer's instructions were scanned with the Illumina microarray platform. Differentially expressed genes were selected based on Significance Analysis of Microarrays criteria: fold change (FC) < 0.6 for downregulated, FC > 1.5 for upregulated; q value < 0.02; and detection probability greater than 0.95 in all samples.

ACCESSION NUMBERS

Microarray and ChIP-seq data are accessible at the GEO database under accession numbers GSE19023 and GSE19019, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Experimental Procedures, three figures, and three tables and can be found with this article online at doi:10.1016/j.stem.2009.12.009.

ACKNOWLEDGMENTS

We are grateful to the Biomedical Research Council, Agency for Science, Technology and Research and Singapore Stem Cell Consortium for funding. J.H. is supported by the NUS Graduate School for Integrative Sciences and Engineering Scholarship. J.-H.N. is supported by the A*STAR graduate scholarship. We thank Xiangling Ng and Kuee-Theng Kuay for technical support and Meng-Chun Hu for plasmids. We acknowledge Valere Cacheux-Rataboul for karyotype analysis and GTB group for sequencing. We thank Andrew Hutchins and Felicia Huimei Hong for comments to the manuscript.

Received: October 20, 2009
Revised: November 28, 2009
Accepted: December 14, 2009
Published online: January 21, 2010

REFERENCES

Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., Jiang, J., et al. (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell *133*, 1106–1117.

Feng, B., Jiang, J., Kraus, P., Ng, J.H., Heng, J.C., Chan, Y.S., Yaw, L.P., Zhang, W., Loh, Y.H., Han, J., et al. (2009a). Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat. Cell Biol. *11*, 197–203.

Feng, B., Ng, J.H., Heng, J.C., and Ng, H.H. (2009b). Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. Cell Stem Cell *4*, 301–312.

Gu, P., Goodwin, B., Chung, A.C., Xu, X., Wheeler, D.A., Price, R.R., Galardi, C., Peng, L., Latour, A.M., Koller, B.H., et al. (2005). Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. Mol. Cell. Biol. *25*, 3492–3505.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K., et al. (2009). A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell *5*, 491–503.

Kim, J.B., Greber, B., Araúzo-Bravo, M.J., Meyer, J., Park, K.I., Zaehres, H., and Schöler, H.R. (2009). Direct reprogramming of human neural stem cells by OCT4. Nature *461*, 649–653.

Lewitzky, M., and Yamanaka, S. (2007). Reprogramming somatic cells towards pluripotency by defined factors. Curr. Opin. Biotechnol. *18*, 467–473.

Maherali, N., and Hochedlinger, K. (2009). Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. Curr. Biol. *19*, 1718–1723.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. *26*, 101–106.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379–391.

Paré, J.F., Malenfant, D., Courtemanche, C., Jacob-Wagner, M., Roy, S., Allard, D., and Bélanger, L. (2004). The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. J. Biol. Chem. *279*, 21206–21216.

Sablin, E.P., Krylova, I.N., Fletterick, R.J., and Ingraham, H.A. (2003). Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1. Mol. Cell *11*, 1575–1585.

Schöler, H.R., Ruppert, S., Suzuki, N., Chowdhury, K., and Gruss, P. (1990). New type of POU domain in germ line-specific protein Oct-4. Nature *344*, 435–439.

Silva, J., Nichols, J., Theunissen, T.W., Guo, G., van Oosten, A.L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., and Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. Cell *138*, 722–737.

Solomon, I.H., Hager, J.M., Safi, R., McDonnell, D.P., Redinbo, M.R., and Ortlund, E.A. (2005). Crystal structure of the human LRH-1 DBD-DNA complex reveals Ftz-F1 domain positioning is required for receptor activity. J. Mol. Biol. *354*, 1091–1102.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Wernig, M., Meissner, A., Cassady, J.P., and Jaenisch, R. (2008). c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell *2*, 10–12.

Yang, F.M., Pan, C.T., Tsai, H.M., Chiu, T.W., Wu, M.L., and Hu, M.C. (2009). Liver receptor homolog-1 localization in the nuclear body is regulated by sumoylation and cAMP signaling in rat granulosa cells. FEBS J. *276*, 425–436.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917–1920.